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Cell Death during Development of Testis and Cerebellum in the Mutant Mouse Weaver

S. M. W. Harrison* and S. K. Roffler-Tarlov*,†

**Program in Cell, Molecular, and Developmental Biology, Department of Anatomy and Cell Biology, and †Department of Neuroscience, Tufts University School of Medicine, Boston, Massachusetts 02111*

The murine mutation *weaver* confers early death during development on cells in testes, cerebellum, and midbrain. The results reported here support the hypothesis that the action of *weaver* is intrinsic to testes and independent of Sertoli cells: germ cells are the only testicular cell type seen to die in weaver homozygotes, while Sertoli cell-dependent development of the blood testis barrier is normal. This report includes characterization of patterns of germ cell death and cerebellar granule cell death in homozygous weavers with respect to that seen during normal development by *in situ* end-labeling of DNA and high-magnification light microscopy. Comparison of the spatial distribution of dying cells in the weaver's cerebellum with that of dividing cells revealed disarray in the external germinal zone. The results show that cells vulnerable to *weaver* die by apoptotic and nonapoptotic mechanisms and indicate that *weaver*-induced cell death is not the consequence of extended naturally occurring developmental cell death, although their timing overlaps. Thus, although the death of cells in each region is likely to be caused by the same mutation, a base pair substitution in the G protein-coupled inwardly rectifying potassium channel 2 gene, the cell death program activated differs depending on cell type.

INTRODUCTION

Weaver (gene symbol, *wv*) causes cell death during development in both testes and brain. *Weaver* is a naturally occurring murine mutation (Lane, 1964; Sidman *et al.*, 1965) in the G protein-coupled inwardly rectifying potassium channel gene, *Girk2* (Patil *et al.*, 1995). In the weaver's testis, the majority of spermatids and many spermatocytes die (Harrison and Roffler-Tarlov, 1994; Vogelweid *et al.*, 1993). Within the brain, degeneration occurs in the midbrain where subsets of the dopamine-containing neurons are vulnerable (Graybiel *et al.*, 1990; Roffler-Tarlov, 1992; Roffler-Tarlov *et al.*, 1996; Smith *et al.*, 1990; Triarhou *et al.*, 1988) and in the cerebellum where enormous numbers of granule cells disappear (Rakic and Sidman, 1973a), as do Purkinje cells and neurons in the deep cerebellar nuclei (Maricich *et al.*, 1997; Smeyne and Goldowitz, 1990). The defects borne by homozygous weavers have observable consequences. Male mice carrying two copies of the mutant gene are sterile and mice of both sexes suffer ataxia, tremor, and hind leg weakness. Heterozygous weavers show less severe cellular defects and no behavioral changes.

This study was prompted by questions about the nature of cell death in the testis of homozygous weavers and of whether the same genetic defect acting in several cell populations initiates the same type of cell death in each. Although it is known that *wv* acts intrinsically in cerebellar granule cells and Purkinje cells, this information is not yet available for any of the other vulnerable cell populations. The results of our study are not definitive on this point regarding the male germ cells, but they provide evidence to suggest that male germ cells are among the direct cellular targets of this mutant gene. Finally, we addressed the question of whether *wv*-induced cell death is related to the naturally occurring developmental death that takes place in these cell groups.

It is well established that cell death is a fundamental component of normal development in many tissues, including the testes and brain, serving to regulate cell number in accordance with the functional maturation of the tissue (Oppenheim, 1991). Naturally occurring developmental cell death is thought to result from activation of genetically controlled death programs that eliminate cells through apoptotic or non-apoptotic, non necrotic, but not necrotic mechanisms (Clarke, 1990; Kerr *et al.*, 1972; Kerr and Harmon, 1991; Pilar and Landmesser, 1976). Specific sets of morphological and biochemical characteristics define each mechanism of death. Apoptotic cell death is identified by a collection of traits including cell shrinkage due to condensation of the cytoplasm, pyknotic nuclei, cleavage of the cellular DNA into nucleosome-sized fragments, and disintegration of the cell into discrete membrane-bound vesicles

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(Kerr *et al.*, 1972). Non-apoptotic, non-necrotic deaths show only some of the features of apoptosis in addition to morphologies that are not seen in apoptotic cells, such as autophagic vacuoles and evidence of endocytosis (Clarke, 1990 for review). Necrosis is distinguished by osmotic swelling and rupture of the cell and its organelles leading to large regions of tissue damage (Buja *et al.*, 1993). Naturally occurring death in male germ cells takes place by apoptotic mechanisms in meiotic cells (Allan *et al.*, 1992; Billig *et al.*, 1995) and by non-apoptotic mechanisms in postmeiotic cells (Russell *et al.*, 1987; Russell *et al.*, 1990). Naturally occurring cell death among the dopamine-producing neurons in the midbrain's substantia nigra is apoptotic (Janec and Burke, 1993; Oo *et al.*, 1996). The initial period of naturally occurring developmental cell death in the granule cells is apoptotic followed by a period of non-apoptotic death (Caddy and Biscoe, 1979; Wood *et al.*, 1993).

Pathological cell death in weaver mice overlaps and extends beyond the normal period of developmental death in all of the vulnerable cell populations suggesting *wv* may interfere with the regulation of this normal process. We report the results of a comparison of the morphology and the spatial characteristics of *wv*-induced cell death with that seen during normal development. Cell death in the testis and cerebellum of homozygous weaver mice and wild-type controls was examined using standard histological techniques and *in situ* end-labeling of DNA fragments generated during apoptosis.

MATERIALS AND METHODS

Mice

All mice used for this study were from a colony established in our animal facility with heterozygous weaver or wild-type breeding pairs purchased from the Jackson Laboratory (Bar Harbor, ME). All mice are on a C57BL6/6I Le-A$^{w\text{-}j}$X CBA/CaGnLeF hybrid background. The mice studied were adults and pups from a range of postnatal ages, and were of three genetic types: wild-type (+/+), heterozygous weaver (+/*wv*) and homozygous weaver (*wv*/*wv*). An individual's phenotype was determined by postmortem examination of its cerebellum and by its demonstration of behavioral characteristics associated with homozygosity at the *wv* locus. The size and shape of the cerebellum distinguishes the phenotype of mice on postnatal-day 7 (P7; P0 is the day of birth) and older. As early as P7, loss of granule cells is profound in the *wv*/*wv* cerebellum which is about one-fifth the normal size with a pronounced sulcus at the midline. Granule cell loss is moderate in +/*wv* mice and the cerebellum is slightly smaller than wild-type, and misshapen and deflated. These differences were used to assign a phenotype to P7-P10 pups. Homozygous weaver mice older than P10 could also be identified by their ataxia, hindlimb weakness, and tremor, behavior that is not apparent in younger *wv*/*wv* pups. Cerebellar sections stained with hematoxylin were used to confirm the initial phenotypic identifications of all mice used in this study. Histologically, the *wv*/*wv* cerebellum is characterized by marked depletion of granule cells in the internal granular cell layer, ectopically placed granule cells in the molecular layer, and disordered placements of Purkinje cells. The +/*wv* cerebellum also has a disorganized Purkinje cell layer and ectopic granule cells, but a less severe reduction in numbers of granule cells.

Initial Tissue Preparation for All Experiments

Mice were deeply anesthetized with Nembutal (40 mg/kg, i.p.). To ensure proper fixation of the testes, mice were transcardially perfused with a 37°C heparin-procaine-saline solution (20 U/ml heparin, 5 mg/ml procaine-HCl, 0.9% NaCl) until the vasculature was cleared (Forssmann *et al.*, 1976; Sprando, 1990). Mice were then perfused with the appropriate fixative for each experiment as described below.

Tissue Preparation for Histology, in Situ End-Labeling, and Bromodeoxyuridine Immunohistochemistry

Mice were perfused with Bouin's fixative until the tissues were well fixed: approximately 50 ml for adults and 25–40 ml for younger mice depending on the animal's size. Testes and brains were removed and placed in Bouin's fixative overnight at room temperature. Tissues were dehydrated, cleared, and paraffin embedded. Ten μm-thick sections were cut, mounted on glass slides, and allowed to dry overnight at 42°C. The sections were deparaffinized, rehydrated, and washed in water just before use. Testicular and cerebellar sections were stained with hematoxylin before morphological examination.

Tissue Preparation for Thin Sections of Testis

Mice were perfused with 5% glutaraldehyde, 0.05 *M* sodium cacodylate (Sprando, 1990). Testes were removed, cut in half transversely, immersed in the same fixative for 2 hours at 4°C, and post-fixed in 1% osmium tetroxide. Testes were embedded in Epon using standard tissue preparation techniques suitable for electron microscopy. One μm-thick sections were stained with toluidine blue.

Tissue Preparation for Examination of the Blood Testis Barrier

Mice were perfused with a hypertonic fixative of 5% glutaraldehyde, 0.05 *M* sodium cacodylate, 10% dextrose (Russell *et al.*, 1990). The testes were removed and immersion fixed in the hypertonic fixative for 2 hours at 4°C. The testes were washed in 0.05 *M* sodium cacodylate buffer for 10 minutes, cut in half transversely, post-fixed in 1% osmium tetroxide, and embedded in Epon following standard procedures. Half-μm-thick sections were stained with toluidine blue.

In Situ End-Labeling of Free DNA Ends

Tissue sections were treated with freshly prepared 40 mg/ml proteinase K at room temperature (7.5 minutes for brain sections, 15 minutes for testis sections). The sections were incubated with 240 U/ml terminal deoxynucleotidyl transferase and 20 m*M* biotin-14-dCTP in a cacodylic acid buffer at 37°C for 1–2 hours. Sections were washed, blocked with 2% BSA, and incubated with streptavidin-b-galactosidase conjugate (1:1000; Boehringer Mannheim) for 30 minutes at room temperature. Incubation followed in 0.075% 5′-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside (X-gal) in dimethyl formamide at 37°C until the reaction product was visible.

Sections were counterstained with nuclear fast red. Positive and negative controls accompanied each set of experimental sections. Positive control sections were treated for 15 minutes with 40 U/ml RNase-free DNase at 37°C following proteinase treatment. Negative control sections were incubated without terminal transferase during the end-labeling reaction. The method is based on that of Gavrieli *et al.* (1992).

Bromodeoxyuridine (BrdU) Treatment and Detection of Mitotic Cells

Mouse pups were injected with 5-bromodeoxyuridine in 0.9% NaCl, 0.007 *N* NaOH (100 mg/g, i.p.) (Herrup and Busser, 1995; Miller and Nowakowski, 1988). Approximately two hours after BrdU injection, pups were sacrificed and their tissues embedded in paraffin as described above. Ten-micron-thick sagittal cerebellar sections were deparaffinized, rehydrated, and incubated with 2 *N* HCl for 30 minutes (Herrup and Busser, 1995). Sections were incubated with anti-BrdU antibody (1:100; Becton Dickenson) overnight at 4°C and then with fluoresceinated anti-mouse IgG antibody (1:200; Sigma) for 2 hours at room temperature.

Generation of Images

Images in this report were either captured digitally, or photographically on color slide film and scanned into a computer using a 35 mm slide scanner. All images were processed in Adobe PhotoShop and represent the images observed with a microscope as closely as possible.

RESULTS

Morphology of Cell Death in the Weaver's Testis

Normal spermatogenesis occurs in overlapping waves within the seminiferous tubules generating a highly ordered germinal epithelium that consists of three or four layers of germ cells, each at a different stage of development. A cross section of a normal tubule reveals a peripheral layer of mitotic germ cells (spermatogonia), a medial layer of meiotic germ cells (spermatocytes), and one or two internal layers of haploid postmeiotic germ cells (round and elongated spermatids) that surround the lumen of the tubule. This organization is disrupted in the weaver's seminiferous tubules by the death of germ cells (Harrison and Roffler-Tarlov, 1994; Verina *et al.*, 1995; Vogelweid *et al.*, 1993). The severity of germinal epithelial degeneration differs greatly between individual seminiferous tubules within a single testis in each of the adult homozygous weaver (*wv*/*wv*) mice that we have examined ($n = 12$, ranging in age from 6 weeks to 1 year) (this study; Harrison and Roffler-Tarlov, 1994). A striking example of the variability within an individual weaver's testis is shown in Figure 1. Some tubules (Fig. 1a) had few dying cells and showed concentric organization characteristic of the wild-type tubule (Fig. 1A), although the number of elongated spermatids was reduced compared to wild-type. In other tubules (Fig. 1a′), there were many abnormal cells that displayed characteristics associated with cell death and elongating spermatids were absent. The most severely affected tubules (Fig. 1a″) contained no developing germ cells but retained spermatogonial stem cells and Sertoli cells at their periphery.

The features of dying cells in the *wv*/*wv* germinal epithelium were compared to those of germ cells that die during normal development in the normal testis. Deaths were identified as apoptotic, non-apoptotic, or necrotic based on attributes of the cell, such as condensed or pyknotic nuclei which are indicative of apoptotic and non-apoptotic deaths, and on the cell's chromatin conformation, as judged by the presence of enzymatically labeled cellular DNA fragments typical of apoptotic but not non-apoptotic cell deaths. Necrotic cells were not seen in the testes of *wv*/*wv* nor +/+ mice.

Dying germ cells in the seminiferous tubules of three month-old wild-type (+/+) mice were scarce and pervasive degeneration of the seminiferous epithelium was never seen. The few degenerating +/+ germ cells seen were cells with highly condensed and darkly stained chromatin and *in situ* end-labeled cells. Cells with pyknotic nuclei were found at all levels within the epithelium, however, the end-labeled cells lay primarily in small clusters toward the periphery of the tubule (Fig. 1B). These cells likely represent a syncytially-linked clone of primary spermatocytes undergoing apoptosis. Wild-type germ cells at more advanced stages of development were not end-labeled in these studies, although, round spermatids with pyknotic nuclei were found in some tubules (data not shown). In contrast, numerous *in situ* end-labeled cells were present in the seminiferous tubules of the adult *wv*/*wv* mouse (Fig. 1b) indicating extensive germ cell loss through an apoptotic mechanism. The end-labeled cells were found scattered throughout the germinal epithelium and within the lumen of the tubule. The size variation among the labeled entities suggests that some were chromatin-containing cell fragments, or apoptotic bodies, formed when the cell disintegrated during the final stages of apoptosis. Although the weaver's seminiferous epithelium was highly disorganized, examination of cell morphology and cell associations indicate that many of the *in situ* end-labeled cells were pachytene primary spermatocytes. Numerous *in situ* end-labeled cells were present in the seminiferous tubules of the adult *wv*/*wv* mouse (Fig. 1b) indicating extensive germ cell loss through an apoptotic mechanism. The end-labeled cells were found scattered throughout the germinal epithelium and within the lumen of the tubule. The size variation among the labeled entities suggests that some were chromatin-containing cell fragments, or apoptotic bodies, formed when the cell disintegrated during the final stages of apoptosis. Although the weaver's seminiferous epithelium was highly disorganized, examination of cell morphology and cell associations indicate that many of the *in situ* end-labeled cells were pachytene primary spermatocytes. Some dying *wv*/*wv* germ cells also showed condensed dark-staining nuclei (Fig. 1a′) and many *wv*/*wv* germ cells exhibited a striking death morphology, not seen in the wild-type testis, that consisted of swollen nuclei, and an apparent lack of nuclear and cytoplasmic contents (Fig. 1a′). These cells were not end-labeled (Fig.

1b). This combination of features provides evidence for the activation of a non-apoptotic death mechanism in a population of *wv*/*wv* germ cells. Cellular morphology and position within the epithelium indicate that many of these cells were round spermatids. Cells in mitotic and meiotic metaphase, identified by their large size, uniform shape, and the presence of a mitotic or meiotic spindle, were labeled in both +/+ and *wv*/*wv* seminiferous tubules (Fig. 1B, 1b). This may be due to an increased accessibility of free single-stranded DNA ends during metaphase (Ansari *et al.*, 1993) or these cells may be dying an apoptotic death.

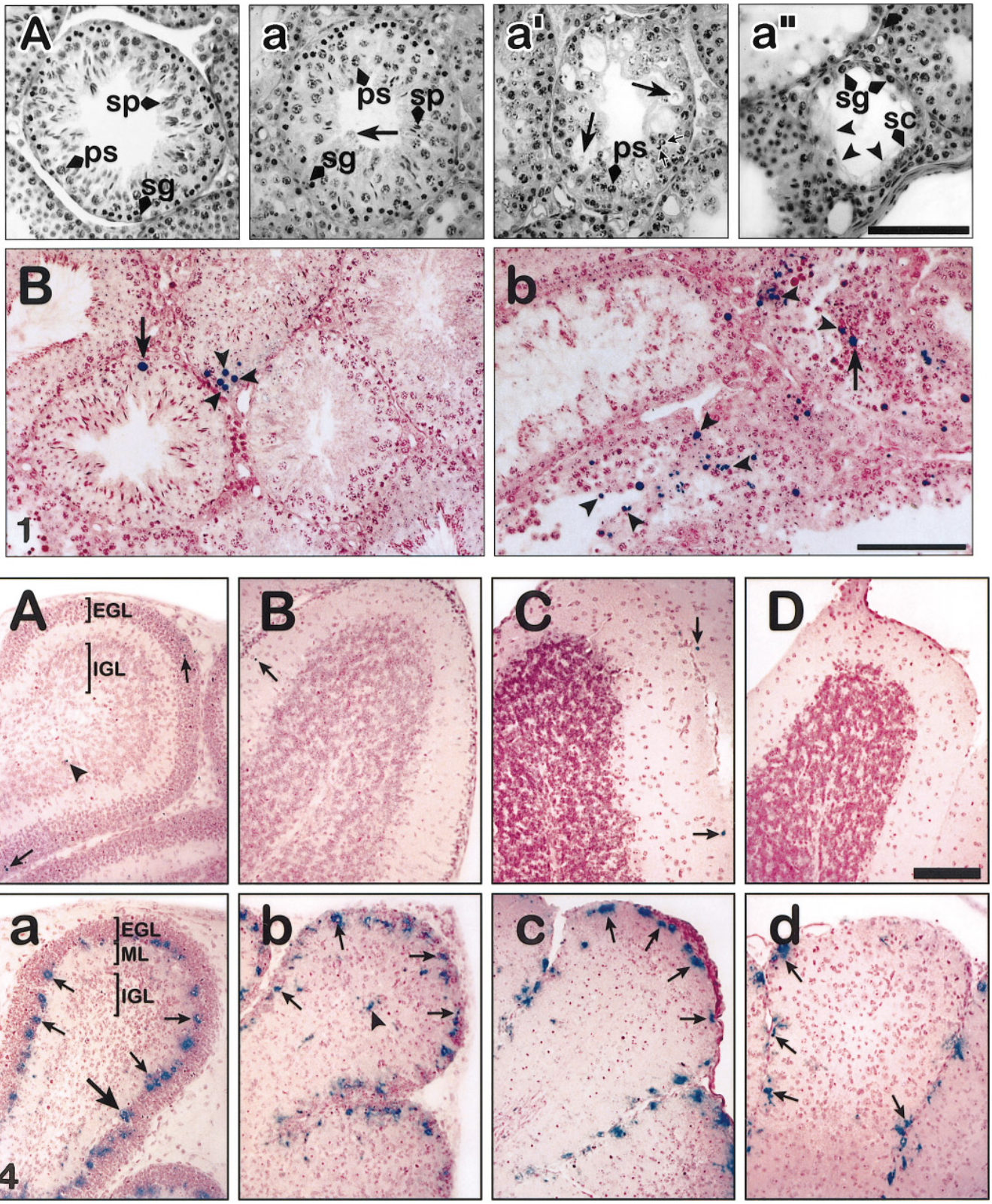
To examine the morphology of individual dying cells in the *wv*/*wv* testis more closely, one μm-thick plastic-embedded sections of adult *wv*/*wv* testes were stained with toluidine blue and studied using high magnification light microscopy. Figure 2 shows a representative seminiferous tubule from a three month-old *wv*/*wv* testis. Several cells within this tubule had the darkly stained, vacuolated cytoplasm, and heavily stained clumps of chromatin that are indicative of apoptotic cell death (Kerr *et al.*, 1972). The morphology of these cells supports the finding from the *in situ* end-labeling study that many *wv*/*wv* germ cells die by apoptosis. Cells displaying swollen morphology were also in this tubule. The cytoplasm was uniformly and lightly stained, and the nuclear membrane seemed intact but the darkly stained chromatin clumps seen in apoptotic cells were absent, giving these cells a ghost-like appearance.

The testicular pathology associated with the *weaver* mutation appears between P21 and P28 (Harrison and Roffler-Tarlov, 1994). To determine whether there is an increased number of apoptotic cells in the weaver's testis throughout the first round of spermatogenesis, prior to overt degeneration of the seminiferous epithelium, *in situ* end-labeling reactions were carried out on testicular sections from P7, P14, P21, and P28 +/+ and *wv*/*wv* mice. The results showed few apoptotic cells in testes of both +/+ and *wv*/*wv* mice on P7, P14, and P21 (data not shown). However, a greater number of *in situ* end-labeled cells were present in the seminiferous epithelia from P28 *wv*/*wv* mice than from P28 +/+ mice and these cells were found primarily in degenerating tubules (data not shown).

The integrity of the germinal epithelium and the status of spermatogenesis was also assessed in heterozygous weaver (+/*wv*) to establish whether the testicular degeneration associated with *weaver* appeared in +/*wv* males. Heterozygous weavers studied at P7, P21, P28, P35, and P90 showed no evidence of abnormal spermatogenesis (data not shown). To determine if development of a testicular phenotype was delayed in +/*wv* mice and if it worsened over time in *wv*/*wv* mice with time, we compared the morphology of aged +/+, +/*wv*, and *wv*/*wv* seminiferous tubules and epididymides. Heterozygous weavers were indistinguishable from age-matched wild-types at all ages examined between 3 and 18 months, and *wv*/*wv* males up to 14 months old did not suffer any greater degeneration of their seminiferous epithelia than younger adult males of the same genotype (data not shown).

FIG. 1. Degeneration and apoptotic death of spermatogenic cells in adult *wv*/*wv* mice. (A, a) Photomicrographs of hematoxylin-stained transverse sections through seminiferous tubules from wild-type (+/+) and homozygous weaver (*wv*/*wv*) mice illustrate the range of germinal epithelial degeneration in an adult *wv*/*wv*. (A) A seminiferous tubule from a three month-old +/+ mouse. Mitotic stem cells (spermatogonia, sg) line the basal aspect of the tubule, meiotic primary spermatocytes (ps) are present in the medial portion, and differentiating spermatids (sp) surround the lumen of the tubule. (a, a′, a″) Seminiferous tubules from a single three month-old *wv*/*wv* mouse display a range of phenotypes. (a) A little affected tubule. Completely unaffected tubules are rare in the *wv*/*wv* testis. Most show some sign of degeneration such as the small cluster of dying cells present in this tubule (arrow). (a′) In more affected *wv*/*wv* tubules, elongating spermatids are absent. Some degenerating round spermatids display condensation and nuclear margination of chromatin (small arrows), whereas others are engulfed in large vacuolar-like areas (large arrows). The dying cells are dispersed among many primary spermatocytes (ps) that appear normal. a″: The most severely affected tubules lack most cells, although spermatogonia (sg) and Sertoli cells (sc) are usually retained in the normal location at the periphery of the tubule and the Sertoli cell's cytoplasmic extensions (arrowheads) form a diffuse halo around the lumen of the tubule. Scale bar in a, 100 μm. (B, b) Photomicrographs of apoptotic cells, in transverse testicular sections from 35 day-old +/+ and *wv*/*wv* mice identified by *in situ* end-labeling of free DNA-ends. (B) Wild-type seminiferous tubules contain a few *in situ* end-labeled cells (arrowheads) which usually lie in small clusters at the periphery. Metaphase cells (arrow) often stain but are distinguished from apoptotic cells by size and mitotic or meiotic spindle. (b) The *wv*/*wv* seminiferous tubules contain many *in situ* end-labeled cells or cell fragments (arrowheads) scattered throughout the germinal epithelium. Metaphase cells in *wv*/*wv* mice are also labeled (arrow). Scale bar in b, 200 μm.

FIG. 4. Apoptotic cell death in developing wild-type (+/+) and homozygous weaver (*wv*/*wv*) cerebella. Photomicrographs of *in situ* end-labeled cells in saggital sections from +/+ (A, B, C, D) and *wv*/*wv* (a, b, c, d) cerebella on postnatal (P) days-7, 14, 21, and 28 respectively. (A, a) On P7 a wide band of granule cells is contained in the external germinal layer (EGL) in both +/+ (A) and *wv*/*wv* (a) mice. The +/+ EGL (A) contains few, scattered end-labeled cells (arrows) and occasionally an end-labeled cell (arrowhead) is seen in the internal granule layer (IGL). The EGL of the *wv*/*wv* mouse (a) contains many heavily end-labeled cells (small arrows) which lie along the border of the EGL and the developing molecular layer (ML) and are often found in small clusters. (B, b) On P14 the EGL of both +/+ (B) and *wv*/*wv* (b) cerebella is much narrower. The pattern of *in situ* end-labeled cells is similar to that seen on P7 for each genotype: few cells (arrow) are stained in the +/+ cerebellum whereas many cells (arrows) are intensely stained in the *wv*/*wv* cerebellum. In addition, heavily stained cells (arrowheads) are present in the *wv*/*wv* IGL. (C, c) The EGL is depleted in the P21 +/+ cerebellum (C) and only a few cells (small arrows) within the ML are end-labeled. A very thin EGL persists in the *wv*/*wv* cerebellum (c) where end-labeled cells (large arrows) are abundant along its internal margin. (D, d) End-labeled cells are rare in the P28 +/+ cerebellar cortex (D). The *wv*/*wv* EGL (d) is not obvious on P28, however, a large number of cells (arrows) along the pial surface are end-labeled. Scale bar, 100 μm.



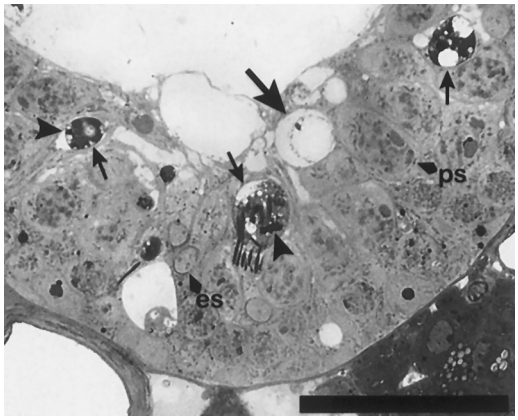


FIG. 2. High magnification photomicrograph of degenerating germ cells in the seminiferous epithelium of an adult homozygous weaver (*wv*/*wv*) mouse. A 1 μm-thick toluidine blue-stained transverse section through a three month-old *wv*/*wv* plastic embedded testis shows several cells that exhibit morphological characteristics of apoptosis. These cells contain darkly stained clumps of fragmented chromatin (arrowheads) and numerous vacuoles (small arrows) resulting from cytoplasmic condensation. Other degenerating cells (large arrow) display expanded plasma and nuclear membranes but little cytoplasm or nucleoplasm, suggestive of non-apoptotic, non-necrotic death. Dying cells of both types are intermingled with normal germ cells (primary spermatocytes, ps; elongating spermatids, es). Scale bar, 50 μm.

No +/*wv* mice older than eighteen months or *wv*/*wv* mice older than 14 months were examined.

Development and Maintenance of the Blood Testis Barrier in Weaver Seminiferous Tubules

The cellular target of *wv* that leads to male sterility has not been identified. In addition to male germ cells, candidate target populations include those that compose the hypothalamic-pituitary-gonadal endocrine axis. Sertoli cells are one of the group of mediators of information from this axis. To control the intratubular environment for germ cell development, Sertoli cells establish and maintain a blood testis barrier (BTB) that is impermeable to most molecules. Adjacent Sertoli cells form specialized junctional complexes between their cytoplasmic extensions, confining spermatogonia outside the barrier along the basal aspect of the tubule and harboring meiotic and postmeiotic germ cells on the luminal side of the barrier. We examined the development and integrity of the BTB to assess the functional status of Sertoli cells in weaver mice. Abnormal barrier formation would suggest that *wv* acts in Sertoli cells, or in another cell type that is part of the hypothalamic-gonadal axis, and indirectly causes germ cell death.

The barrier can be visualized in testes fixed with a dextrose-containing hypertonic fixative (Russell *et al.*, 1990). Cells directly exposed to the fixative, such as those on the basal side of the barrier, suffer from the osmotic effects of the dextrose and shrink, leaving large intercellular gaps. Because dextrose can not traverse the BTB, cells on the luminal side are protected from the hypertonicity of the fixative and maintain close apposition with one another. If the barrier is not intact, cells throughout the cross section of the tubule shrink away from each other.

The BTB is normally established by Sertoli cells as the first germ cells enter meiosis (Russell, 1978; Russell *et al.*, 1989) which occurs around P8 in the mouse. The development and integrity of the BTB were examined in 0.5 μm-thick testicular sections from P8, P10, and adult +/+ and *wv*/*wv* mice perfused with hypertonic fixative. Our results show that most +/+ and *wv*/*wv* P8 tubules contained shrunken cells throughout the tubular cross section (Figs. 3A, 3a). Only a small number of tubules had the fixative-induced shrinkage pattern consistent with formation of the BTB. By P10 the BTB shrinkage pattern was evident in a majority of the seminiferous tubules of both +/+ (Fig. 3B) and *wv*/*wv* (Fig. 3b) testes. There was no delay in formation of the BTB in *wv*/*wv* mice. In addition, the BTB was maintained in the adult *wv*/*wv* testes (Fig. 3c) despite the degeneration of adjacent seminiferous epithelium in these tubules.

Morphology, Location, and Persistence of Cell Death during Postnatal Development in the Weaver's Cerebellum

The morphological and spatial characteristics of cell death were examined in cerebella of postnatal-day (P) 7, P14, P21, and P28 +/+ and *wv*/*wv* mice to compare naturally occurring developmental cell death with that induced by the mutation. *In situ* end-labeling reactions carried out on sagittal sections from the vermis of each cerebellum pointed to little apoptotic cell death in P7 and P14 +/+ mice as illustrated in Figs. 4A and 4B. The end-labeled cells detected in these mice were usually found scattered within the external germinal layer (EGL) but a few were present in the internal granular layer (IGL). End-labeled cells were rarely seen in the older +/+ mice (Figs. 4C and 4D): those found in the P21 and P28 cerebella appeared to be randomly distributed throughout the molecular layer and the IGL.

In contrast, numerous *in situ* end-labeled cells were present in the EGL of the homozygous weaver's cerebellum at all ages examined (Fig. 4a–4d). In many cells, β-galactosidase staining was coincident with pyknotic nuclei, a further indication that these cells die by apoptosis. β-galactosidase-positive cells were also found in the *wv*/*wv* IGL where they outnumbered those found in the +/+ IGL at each age, although the prevalence of stained cells in the *wv*/*wv* IGL diminished with age. The apoptotic cells in the P7 (Fig. 4a) and P14 (Fig. 4b) weaver's cerebella lay predominantly along the inner margin of the EGL where postmitotic, premigratory granule cells are positioned in normal mice at these ages. By P21 the EGL had disappeared in the +/+ cerebellum (Fig. 4C), however, it was thin and still visible in both the P21 (Fig. 4c) and P28 (Fig. 4d) *wv*/*wv* cerebella, and the majority of end-labeled cells were confined to this region.

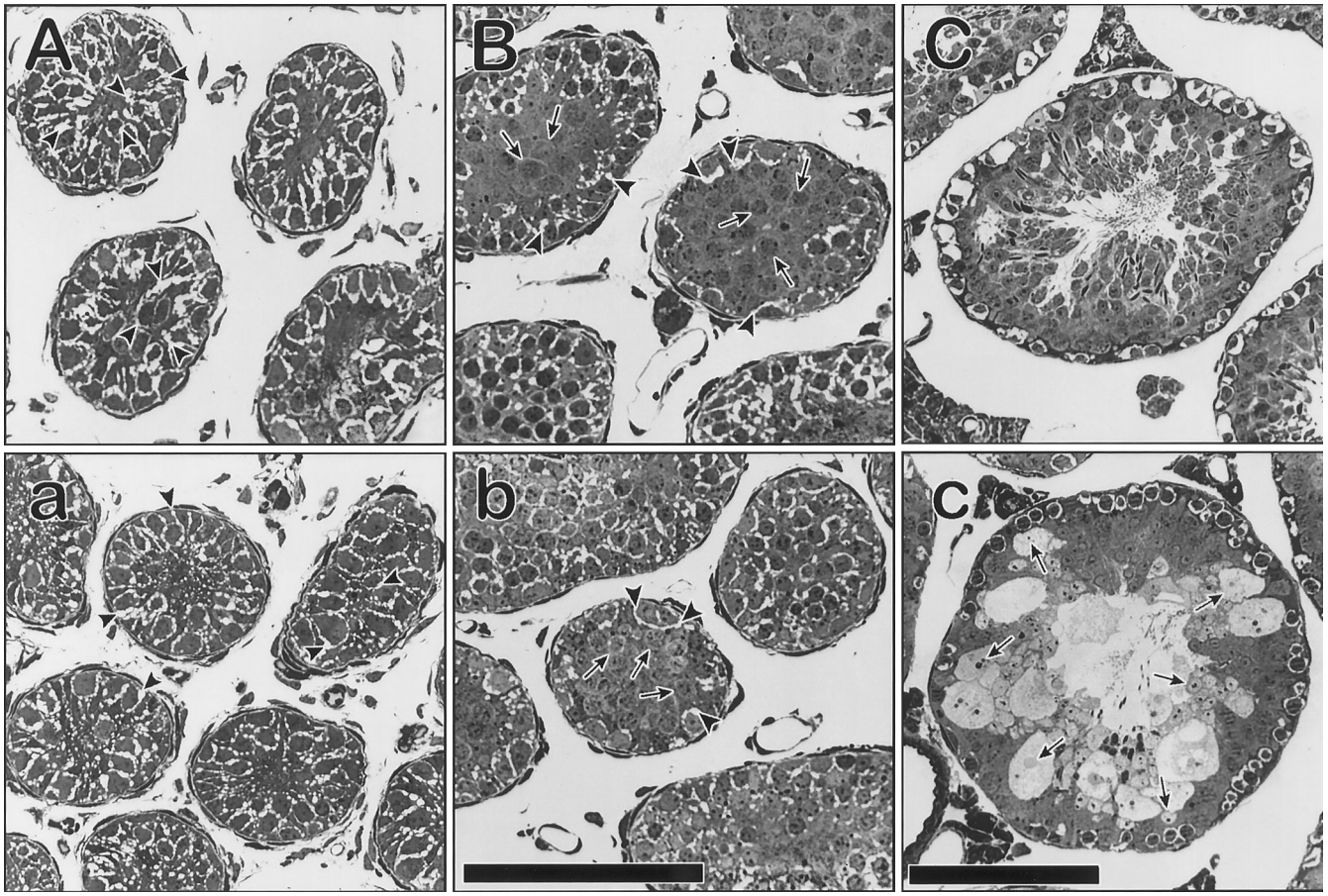


FIG. 3. Postnatal development and maintenance of the blood-testis barrier (BTB) in wild-type (+/+) and homozygous weaver (*wv*/*wv*) mice. Photomicrographs of toluidine blue-stained, 0.5 μm-thick, plastic-embedded transverse sections of +/+ (A, B, C) and *wv*/*wv* (a, b, c) seminiferous tubules following fixation with hyperosmotic fixative. (A, a) The majority of seminiferous tubules in the postnatal (P) day-8 +/+ (A) and *wv*/*wv* (a) mice have not established a BTB indicated by the open space network (arrowheads) created by fixative-induced shrinkage of cells throughout the tubules. There is no visible difference between +/+ and *wv*/*wv* testes. (B, b) On P10, most of the seminiferous tubules in both +/+ (B) and *wv*/*wv* (b) testes have formed a BTB indicated by confinement of cell shrinkage (arrowheads) to the mitotic and premeiotic cells at the periphery. The adluminal meiotic cells (arrows) are protected from fixative-induced condensation, maintaining normal morphology and cellular contacts. No differences are seen between +/+ and *wv*/*wv* mice. (C, c) The BTB is maintained in the seminiferous tubules of the adult +/+ (C) mouse as well as in those of the adult *wv*/*wv* (c) mouse, even in tubules where the seminiferous epithelium is degenerate and contains dying cells (arrows). Scale bar in b, 100 μm for A, a, B, and b. Scale bar in c, 100 μm for C and c.

Organization of Proliferative and Nonproliferative Granule Cells in the Weaver's EGL

The EGL of the mouse cerebellar cortex is subdivided into a peripheral zone of proliferating granule cells and an internal zone of differentiating, but nonproliferative, granule cells (Fujita *et al.*, 1966; Miale and Sidman, 1961). To determine if the weaver's EGL maintains this architecture in the face of extensive granule cell loss along its border with the molecular layer, proliferative cells in the cerebellum were labeled with single pulse of bromodeoxyuridine (BrdU). The P7 +/+ cerebellum (Fig. 5A) showed a band of BrdU-labeled cells along the external half of the EGL, whereas cells in the internal half of the EGL were not labeled. These populations represent the expected dual granule cell zones, proliferative and nonproliferative, in the EGL of the developing cerebellum. Cells in the IGL were rarely labeled in the P7 +/+ cerebellum. The P7 *wv*/*wv* EGL failed to maintain the normal organization of granule cells into two distinct regions. BrdU-labeled cells were found scattered throughout the thickness of the EGL (Fig. 5B). Less heavily labeled BrdU-positive cells were more prevalent in the *wv*/*wv* IGL than in the +/+ IGL.

DISCUSSION

The murine mutation *weaver* compromises the viability of seemingly unrelated cell populations that display many

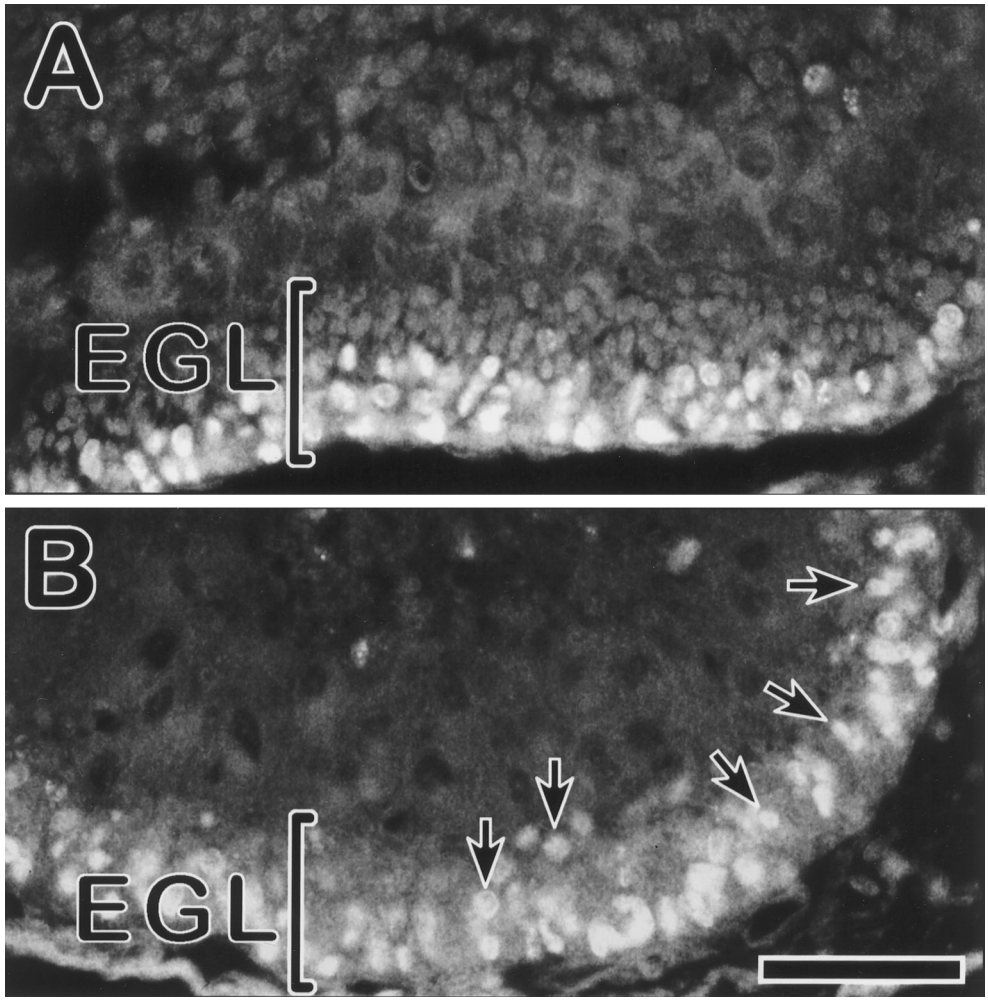


FIG. 5. Localization of dividing cells in the external germinal layer (EGL) in P7 wild-type (+/+) and homozygous weaver (*wv*/*wv*) mice. Photomicrographs of mitotic granule cells, labeled with fluorescein-conjugated anti-BrdU antibodies, +/+ (A) and *wv*/*wv* (B) cerebellar cortices, 2 hours after IP injection of BrdU. The +/+ EGL (A) displays two distinct subdivisions: a peripheral layer of brightly-labeled mitotic granule cells and an internal layer of unlabeled postmitotic granule cells. The *wv*/*wv* EGL (B) does not display discrete subdivisions: BrdU-labeled granule cells (arrows) reside throughout the EGL. Scale bar, 150 μm.

similar responses to the mutation. Each fails to complete differentiation, and subsequently dies during a period that overlaps and extends beyond the period of developmentally mandated death normally seen. In spite of the many similarities in the effects of *wv* among the vulnerable cells, the results of the present studies of *wv*-induced cell death in the cerebellum and the testes, combined with results of similar studies of the weaver's midbrain (Oo *et al.*, 1996), show that *wv* triggers diverse death mechanisms. The mechanism activated is cell type specific, is both apoptotic and nonapoptotic, and may be related to the maturity of the cell at the time it is affected by the mutation. How the mutation in the G protein-gated inwardly rectifying postassium channel (Girk2) that is almost certainly *weaver*, results in cell death is unknown. Channel activation in weaver granule cells, the only weaver cell type examined physiologically to date, reveals reduced inwardly rectifying potassium currents compared to wild-type (Kofuji *et al.*, 1996; Lauritzen *et al.*, 1997; Surmeier *et al.*, 1996). Although not all of the electrophysiological studies agree, several report that the mutated channel is unselective, admitting sodium, a condition that depolarizes cells making them vulnerable to calcium entry and death (Kofuji *et al.*, 1996; Navarro *et al.*, 1996; Silverman *et al.*, 1996; Slesinger *et al.*, 1996; Tong *et al.*, 1996). Such a scenario resembles excitotoxic cell death which is also associated with variable death morphologies that appear to be related to the age of the neurons at the time of insult. For example, Portera-Cailliau *et al.* (1997a; 1997b) have shown that kainic acid administration causes a spectrum of death morphologies, from apoptotic to necrotic, within populations of neonatal rat neurons, whereas adult neurons primarily display morphology

that combines features of apoptosis and necrosis (Ferrer *et al.*, 1995; Pollard *et al.*, 1994). Male germ cells also show distinct death morphologies in response to toxins or hormone deprivation depending on their stage in the seminiferous cycle, whether they are pre or postmeiotic, and the age of the animal (Billig *et al.*, 1995; Wong and Hruban, 1972). Why *weaver* incites cell death in specified cell types during their development remains to be determined but the morphological variations seen among the dying cells are likely to reflect their state of differentiation rather than the ancestral cause, the channel mutation.

Cell Death in the Testis

The germ cell was the only type of dying cell detected in our study in the weaver's testis. We saw that germ cells in the weaver's testes die by two mechanisms: one that leads to an apoptotic morphology detected by *in situ* end-labeling staining and one that does not. The majority of apoptotic deaths occurred in primary spermatocytes in the midst of their first meiotic division. A non-apoptotic death morphology was primarily found in degenerating postmeiotic haploid round spermatids. Somatic cells in the weaver's testes, including Sertoli and Leydig cells, were not detected by *in situ* end-labeling nor did they show overt morphological characteristics of any type of cell death at the resolution used for this analysis. This is in contrast to reports of altered morphology and staining properties of some Sertoli cells detected by EM in older weavers (Verina *et al.*, 1995; Vogelweid *et al.*, 1993). In fact, our examination of aged *wv*/*wv* mice reveals that the demolition of the *wv*/*wv* seminiferous epithelium stops short of eradicating the two cell populations that found the epithelium: the spermatogonial stem cells and the Sertoli cells. It is known that while these cells are present, functional, and receiving the appropriate signals, they will initiate spermatogenic cycles at regular intervals, continuously replenishing the germinal epithelium (Russell *et al.*, 1990). As the aged *wv*/*wv* mice did not show differences in the overall morphology of their testes from younger *wv*/*wv* adults studied previously, we conclude that spermatogenesis is initiated continually in homozygous weavers by spermatogonial stem cells and functional Sertoli cells.

The heterozygote's sperm are a special puzzle. Fifty percent of the postmeiotic spermatids carry the *weaver* mutation, yet the *wv* sperm in the heterozygote undergoes normal development, is released from the testis, and fertilizes eggs. Because cytokinesis is incomplete in meiotic male germ cells, normal haploid sperm and those that carry *wv* share cytoplasm in the heterozygote males that may provide factors that rescue sperm carrying the mutation. Our results indicate that germ cell development in +/*wv* males remains unaffected until at least 14 months of age: neither germ cell death nor seminiferous epithelial degeneration was augmented in these animals when compared to age matched wild-type (+/+) mice. The occasional dying spermatocytes and spermatids seen in the seminiferous tubules and the epididymal lumen in both +/+ and +/*wv* are most likely victims of naturally occurring cell death that serves to prevent abnormal germ cells from completing development (Huckins, 1978; Russell and Clermont, 1977).

A study of the +/*wv* testis by Verina *et al.* (1995) found, as we did, that young adult animals do not present an abnormal testicular phenotype. In contrast to our results, they found a 20% reduction in sperm production and degeneration of the germinal epithelium in 6 month-old +/*wv* males. Although less severe than in *wv*/*wv* males of the same age, the testicular pathology in these +/*wv* males had the same morphological characteristics seen in *wv*/*wv* mice. Our mice and those examined by Verina *et al.* (1995) were maintained on the same hybrid genetic background but subtle differences between the colonies and the extent of inbreeding, in conjunction with the intrinsic variability of the testicular phenotype, may account for the differences between the two studies. The studies agree however, in that the testes of young adult heterozygotes are indistinguishable from those of age-matched normal mice.

Cell Death in the Cerebellum

Our examination of *wv*-induced cell death in the cerebellum revealed that granule cells die by apoptosis, consistent with previous reports (Gillardon *et al.*, 1995; Migheli *et al.*, 1995; Rezai and Yoon, 1972; Smeyne and Goldowitz, 1989; Wullner *et al.*, 1995). In addition, we found that the weaver's EGL is persistent and filled with apoptotic cells until at least P28, whereas the EGL disappears in normal mice much earlier, between P15 and P18 (Fujita *et al.*, 1966). The death of the weaver's granule cells before they have fully differentiated or migrated to their final destination may lead to continuous proliferative signals from granule cell targets, the Purkinje cells. Such an extended period of granule cell production as has been postulated to explain the persistence of the EGL in other granule cell-poor mutants (Herrup and Sunter, 1987; Vogel *et al.*, 1989).

Apoptotic granule cells in the weaver's cerebellum were found in clusters that were nearly always confined to the deepest layer of the EGL bordering the molecular layer at all ages examined. It is in this zone in which postmitotic premigratory neurons are found, separated from the region of proliferating neuroblasts above. However, we saw that in the young weaver's cerebellum, the usual separation of proliferating and postmitotic granule cells in the EGL is disrupted. The BrdU-labeling of mitotic cells in weavers showed that proliferating cells reside throughout the EGL and emphasized the disorder in the EGL of weaver (Rakic and Sidman, 1973b; Smeyne *et al.*, 1991). The consequence of the disorganization seen in the weaver's cerebellum could include alterations in maturity-dependent cell interactions that may be required for normal granule cell differentiation. Observations of wild-type and weaver granule cells *in vitro* and *in vivo* indicate that close apposition of proliferating granule cells may be required for subsequent neurite outgrowth and migration (Gao and Hatten, 1993; Gao *et al.*, 1991) both of which fail in weaver granule cells (Gao *et al.*, 1991; Rakic and Sidman, 1973a; Rezai and Yoon, 1972;

Willinger and Margolis, 1985). Such a scenario would maintain that the altered function of Girk2 would be at the root of the failure to establish order in the weaver's EGL.

Weaver-Induced Cell Death and Naturally Occurring Developmental Cell Death

The temporal proximity of developmental cell death, known to occur in the granule cell, male germ cell, and substantia nigra cell populations, to *wv*-induced cell death suggests that the action of *weaver* may lead to unrestrained naturally occurring developmental cell death. However, the results of our studies show that the *wv*-induced death does not consistently exhibit similarities in its morphology or spatial distribution to developmental cell death.

Developmental cell death takes place during spermatogenesis where it is thought to maintain an appropriate ratio of germ cells to supporting Sertoli cells as well as to eliminate genetically aberrant germ cells (Barr *et al.*, 1971; Huckins, 1978; Oakberg, 1956; Roosen-Runge, 1973; Russell *et al.*, 1987; Russell and Clermont, 1977). Mitotic spermatogonia and young meiotic spermatocytes are the most common victims in this process (Huckins, 1978; Russell and Clermont, 1977). The occasional end-labeled cells that we found in the wild-type testes belonged to these cell populations and their death by apoptosis is consistent with the results of similar studies (Allan *et al.*, 1992; Tapanainen *et al.*, 1993). The increased end-labeling of cells in the weaver's germinal epithelium was also largely confined to the spermatocyte population, however unlike wild-type, spermatocytes in both early and later stages of meiosis were labeled. Degeneration of wild-type germ cells at more advanced developmental stages was rare and these cells, noted because of their aberrant morphology, were not end-labeled (data not shown) comparable to the findings of others (Billig *et al.*, 1995; Mori *et al.*, 1997). Similarly, the death of weaver postmeiotic spermatids was not detected by *in situ* end-labeling, however, the non-apoptotic death morphology of these cells is very different than that seen in normal postmeiotic germ cells; degenerating spermatids appeared swollen and empty in the weaver, but were usually misshapen with condensed nuclei and cytoplasms in the wild-type (data not shown; Russell *et al.*, 1987; Russell and Clermont, 1977).

The pattern of naturally occurring cell death in the wild-type cerebellum observed in this study shares few similarities with that previously documented for the developing mouse cerebellum (Wood *et al.*, 1993). Consistent with the report by Wood *et al.* (1993), we frequently found individual *in situ* end-labeled cells throughout the IGL and both layers of the EGL in young mice, which became less prevalent in older animals and rare after P20. A striking difference between these studies, however, was in the abundance of *in situ* end-labeled cells seen in the cerebella from P7 and P14 mice. The normal mice examined Wood *et al.* (1993) had copious numbers of these cells in both the EGL and the IGL, suggesting that a significant portion of the granule cell population was undergoing apoptosis at any given point throughout the first two postnatal weeks. We found only a few end-labeled cells per section, even in mice at the ages of peak naturally occurring developmental cell death. In addition, Wood *et al.* (1993) noted a correlation in the extent of end-labeling in the EGL and the IGL: regions of the cerebella with a relatively high number of end-labeled cells in the EGL also had a large number of end-labeled cells in the IGL, and vice versa. *In situ* end-labeled cells appeared independently scattered throughout the cerebellar layers in our mice. Our results are similar to the patterns of *in situ* end-labeling in wild-type mouse cerebella reported by others (Wullner *et al.*, 1995; Gillardon *et al.*, 1995).

The *in situ* end-labeling pattern seen in the weaver's cerebellum was easily distinguishable from that in the wild-type mice. Individual end-labeled cells were dispersed in the *wv*/*wv* EGL and IGL similar to the wild-type distribution, but the great majority of end-labeled cells in the weaver's cerebellum lay in heavily stained clusters along the border between the EGL and the molecular layer. This striking *in situ* end-labeling pattern matches that described for pyknotic granule cells in the weaver's cerebellum (Rakic and Sidman, 1973a; Rakic and Sidman, 1973b; Smeyne and Goldowitz, 1989).

Evidence for Intrinsic Weaver Activity in Male Germ Cells

The results of studies using chimeric mice and cerebellar cell cultures indicate that *wv* acts autonomously in cerebellar granule and Purkinje cells (Goldowitz, 1989; Goldowitz and Mullen, 1982; Hatten *et al.*, 1986; Smeyne and Goldowitz, 1990). Such evidence has not been established for the male germ cells. Failure of spermatogenesis in the weaver mouse could result from an intrinsic effect of the mutation on the germ cells or could be due to aberrant *wv* activity in any of the cell populations that form the hypothalamic-pituitary-gonadal axis including Sertoli and Leydig cells in the testis, gonadotropin producing cells in the pituitary, and hypothalamic neurons that regulate pituitary activity. Defects in any of these somatic cell groups could lead alterations in hormonal support required for germ cell development.

Many components of the male reproductive system, in addition to germ cells, require gonadotropins for normal growth, development, and function and, therefore, reflect the health of various parts of the hypothalamic-pituitary-testicular system. An accumulation of observations and experimental results by us and others indicate that the failure of spermatogenesis in weaver mice is not due to abnormal production of circulating levels of anterior pituitary or gonadal hormones. We show here that the development and maintenance of the blood-testis barrier is normal in weaver mice. The barrier is formed by Sertoli cells under the influence of follicle stimulating hormone and testosterone (Janecki *et al.*, 1991; Kerr *et al.*, 1993) indicating that *wv*/*wv* Sertoli cells are able to receive and respond appropriately to hormonal signals. Thus, *weaver* does not appear to be a detriment to Sertoli cell function. Growth and development

of the prostate and seminal vesicles are also sensitive to testosterone. These tissues are of normal weight in weaver mice (our unpublished observations; Vogelweid *et al.*, 1993). Female reproduction is dependent on the same hormones required for male fertility. Homozygous weaver females routinely mate and produce litters. Together this evidence suggests that male germ cells are a primary target of the weaver mutation. This conclusion is also supported by *in situ* localization of *Girk2* mRNA in the testes of pubertal and adult wild-type mice (our unpublished data).

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